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MeCP2 preferentially binds to methylated linker DNA in the absence of the terminal tail of histone H3 and independently of histone acetylation

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Abstract Methyl CpG binding protein 2 (MeCP2) is a basic protein that contains a DNA methyl binding domain. The mechanism by which the highly positive charge of MeCP2 and its ability to bind methylated DNA contribute to the specificity of its binding to chromatin has long remained elusive. In this paper, we show that MeCP2 binds to nucleosomes in a very similar way to linker histones both *in vitro* and *in vivo*. However, its binding specificity strongly depends on DNA methylation. We also observed that as with linker histones, this binding is independent of the core histone H3 N-terminal tail and is not affected by histone acetylation.

Keywords: MeCP2; Chromatin; Nucleosome; DNA methylation; Histones; Acetylation

1. Introduction

Methyl CpG binding protein 2 (MeCP2) is a member of the methyl binding domain (MBD) [1] family of proteins that was first identified in 1989 [2]. Mutations of this protein have been shown to be largely responsible for Rett syndrome [3,4], a neurodevelopmental disorder that primarily affects young girls [5]. MeCP2 binds 5′ methylated CpG DNA regions within a chromatin context and can mediate changes to the local chromatin structure through the recruitment of additional *trans*-acting factors [6]. Chromatin is a macromolecular complex composed of histone proteins associated with DNA. The DNA wraps around a core histone octamer [2 (H2A–H2B) dimers + $(H3–H4)_2$ tetramer] in approximately two left-handed turns to form a nucleosome. The nucleosome is the basic repeating unit of chromatin structure. Adjacent nucleosomes are connected by the intervening linker DNA. Higher order chromatin structures are achieved in part by the binding of linker histones to the linker DNA which facilitates compaction of the chromatin fibre [7].

Understanding the constraints imposed by chromatin on the binding of MeCP2 to its targeted methylated DNA sites has proven to be complicated. About 10 years ago, Nan et al. published an interesting paper [8] which characterized the interaction of MeCP2 with chromatinized methylated/unmethylated DNA templates assembled in the presence or absence of linker histones. The results of such work conclusively showed that MeCP2 preferentially binds nucleosome-organized methylated DNA and that MeCP2 was able to displace 40% of histone H1. It was not clear if MeCP2 had any preference for the linker DNA or for the nucleosome organized methylated DNA and also where histone H1 was displaced from. Ensuing research showed that MeCP2's interaction with mononucleosomes was very similar to that of histone H1 and that hinted at a dependence on DNA methylation [9]. More recent *in vitro* work using reconstituted oligo-nucleosome templates for MeCP2 binding under very low ionic strength conditions showed that MeCP2 could produce novel levels of chromatin compaction in the absence of DNA methylation [10]. This has led to the notion that MeCP2 might also act as a global suppressor of gene activity [10,11]. Nevertheless, when the *in vitro* reconstitutions using unmethylated DNA templates [10] were repeated using methylated DNA constructs, a chromatosome-like structure was observed upon MeCP2 binding [12] in agreement with earlier published results [9]. Despite all this biochemical *in vitro* effort, very little is known about the interactions of MeCP2 with chromatin in an *in vivo* setting.

2. Materials and methods

2.1. DNA construct

The (5S-pmbs) DNA templates used in this work were derived from a 207-12 construct of the sea urchin 5S rRNA gene [13]. Upon excision of a 207 bp fragment by digestion with RsaI, the 5S-207 fragment was cloned and PCR-modified to create the preferential methylation binding sites (pmbs) shown in Fig. 1. Pmbs for MeCP2 were created following the consensus sequence criterion defined in [14]. 5S-pmbs0 refers to the construct which contains no pmbs outside of the core histone binding region. The DNA templates were radiolabeled with [γ-^{32}P]ATP using T4 polynucleotide kinase [15]. DNA was incubated with CpG Methyltransferase (M.*Sss*I) (New England Biolabs) for 4 h at 37 °C and the extent of methylation was checked by digestion with HpaII.

2.2. Chromatin preparation

Chromatin from HeLa S3 cells grown in suspension in the presence or absence of 5 mM sodium butyrate was digested with micrococcal nuclease (Worthington) (30 U/mg) in the presence of 50 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM PIPES (pH 6.8) buffer for 15 min at 37 °C and centrifuged immediately to produce a supernatant (fraction SI). The nuclear pellet was subsequently lysed in 0.25 mM EDTA to further generate a supernatant (fraction SE) and a lysis-resistant pellet (fraction P) as described in [16]. The SI supernatant was fractionated by 5–20% sucrose gradients using a Beckman L8-70M ultracentrifuge [16]. MeCP2 amounts were further characterized by Western blotting and native-PAGE analysis.

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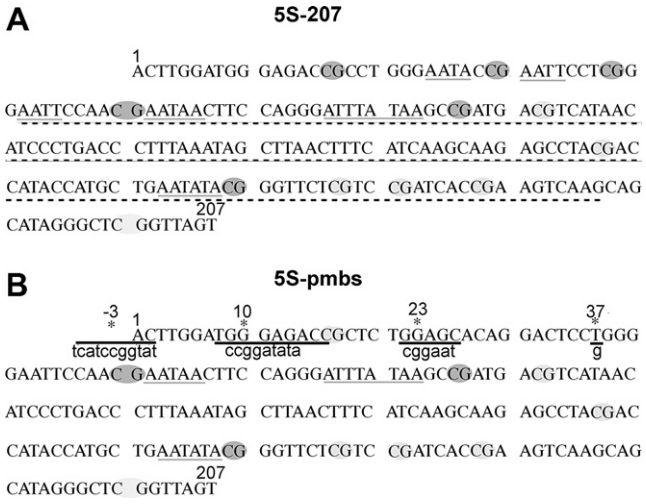

Fig. 1. DNA templates. (A) Nucleotide sequence of the 5S-207 fragment [13]. The dashed black line indicates the main position occupied by the histone octamer when this DNA is reconstituted into nucleosomes [17]. The light- and dark-grey ellipsoids respectively highlight the CpG sites without or with MeCP2 preferential binding [14]. (B) Nucleotide sequence of the 5S-pmbs0 DNA used to generate the different pmbs constructs. The light-grey lines highlight the adjacent CpG $[A/T]_{\geqslant 4}$ consensus regions responsible for the high-affinity binding of MeCP2 [14]. The asterisks and numbers above them point to the position of the newly introduced CpG pmbs using the 5S-207 sequence as a reference for the nucleotide numbering.

2.3. Gel shift assays

2.3.1. DNA. DNA gel shift assays were done by incubating 0.15 pmol of 207 bp DNA and increasing amounts of MeCP2 (see Fig. S1A) for 20 min at 25 °C in a reaction mixture of 10 mM Tris-HCl (pH7.5), 150 μg/ml BSA, 0.1% NP-40, 3 mM DTT, 5% glycerol and 150 mM NaCl and 150 ng of poly(dI-dC):poly(dI-dC).

2.3.2. Nucleosomes. Gel shift assays with nucleosomes were performed by incubating different amounts (see figure legends) of reconstituted mononucleosomes and increasing amounts of MeCP2 for 20 min at 25 °C in the same reaction mixture as above (80 mM NaCl was used in Fig. 5).

3. Results and discussion

3.1. MeCP2 preferentially binds methylated linker DNA in the proximity of the nucleosome

The template DNA used for the nucleosome reconstitutions was a 207 bp DNA segment of the sea urchin 5S rRNA gene (5S-207). This commonly used fragment is a positioning sequence in which the histone octamer predominantly occupies a well-defined position (Fig. 1A) [17–19]. Prior to characterizing MeCP2 binding to reconstituted nucleosomes (Fig. S1D), the binding preference of MeCP2 to both the methylated and unmethylated naked (5S-207) DNA template was assessed (Fig. 2). When incubated with MeCP2 in the absence of competitor DNA, complexes of progressively lower electrophoretic mobility were observed with increasing amounts of MeCP2, both with methylated and unmethylated DNA (Fig. 2A). In the presence of competitor DNA, MeCP2 binding to unmethylated DNA was strongly suppressed, and a series of well-defined complexes was seen with methylated DNA (C1–C4; Fig. 2B). These results conclusively show that in the absence of competitor DNA (Fig. 2A), the MeCP2 binding preference for methylated DNA (Fig. 2B) is completely abolished and MeCP2 binds to both methylated and unmethylated DNA.

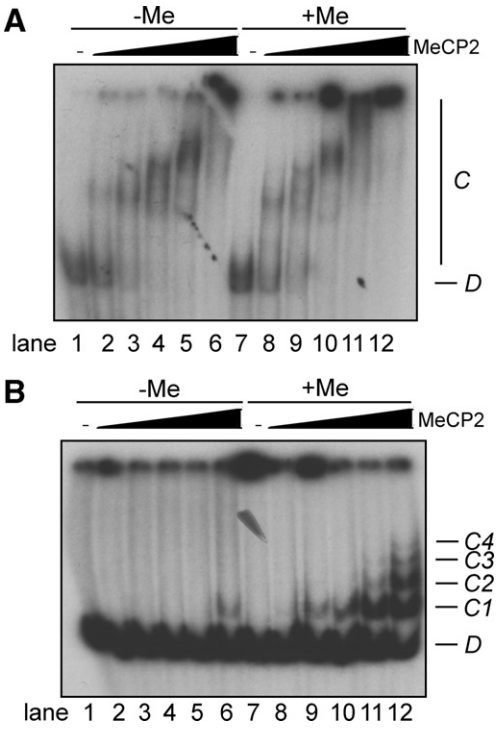

Fig. 2. *MeCP2 preferentially binds to methylated DNA in the presence of competitor DNA.* (A and B) Gel shift assays of the unmodified 207 bp 5S rRNA sequence (5S-207) non-methylated (−Me) or *Sss*I methylated (+Me) in the absence (A) or in the presence (B) of poly(dI-dC):poly(dI-dC) competitor. DNA samples were incubated with 0 (lanes 1 and 7), 0.3 (lanes 2 and 8), 0.6 (lanes 3 and 9), 1.2 (lanes 4 and 10), 2.4 (lanes 5 and 11) and 4.8 (lanes 6 and 12) pmol of MeCP2. *C* and *C1, C2, C3, C4*: MeCP2–DNA complexes arising from binding of MeCP2 to several of the MeCP2 CpG preferential binding sites which are present in the 5S-207 DNA sequence; *D*: free DNA.

This simple experiment brings up an important cautionary note for the interpretation of those studies using (5S rRNA-related) unmethylated DNA templates [10] and underscores the importance of using competitor DNA. The presence of competitor DNA more likely reflects the chromatin environment encountered by MeCP2 in the nucleus. The rather non-specific binding of MeCP2 to methylated or unmethylated DNA in the absence of competitor DNA (Fig. 2A) is merely a reflection of the electrostatically driven interaction between the very basic MeCP2 (pI = 10.0) and DNA. Importantly, DNA in the nucleosome retains many free phosphates as only a very limited number of backbone phosphates interact with the core histones [20–22]. Therefore, all the subsequent nucleosome experiments described in this work were done using (radiolabeled) DNA sequence-defined nucleosomes simultaneously reconstituted with a 10-fold excess of background (cold) 146 bp random sequence nucleosomes [23].

Fig. 3B–D shows that MeCP2 prefers binding to methylated target domains [14] that occur in the linker domain outside of the DNA region protected by the histone octamer. Similarly to naked DNA (Fig. 2B), although less pronounced, it took greater amounts of MeCP2 to shift unmethylated nucleosomes than its methylated counterpart (compare lane 7 with lanes 13, 14 in Fig. 3B and lanes 5, 6 with lanes 9–12 in Fig. 3C). Furthermore, the shift took place at lower MeCP2 concentrations when the pmbs was located within the nucleosomal DNA outside of the preferential histone octamer position (compare

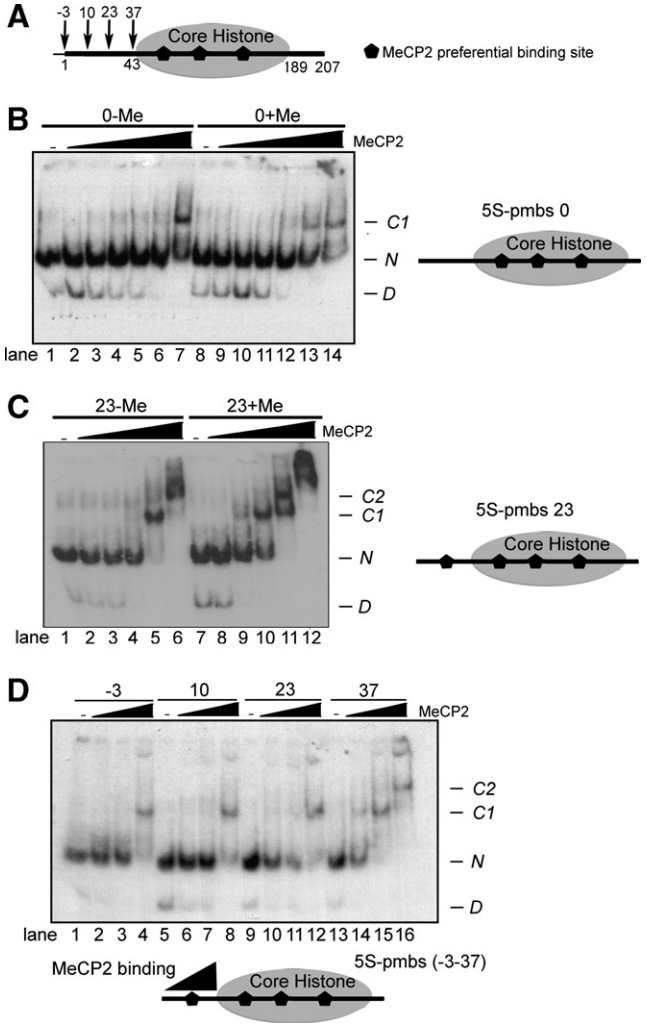

Fig. 3. MeCP2 preferentially binds to the methylated linker DNA region closest to the nucleosome. (A) A cartoon representation of the major position occupied by the histone octamer in the 5S rRNA gene sequence [18,19,36]. (B) Gel shift assay of nucleosomes reconstituted onto unmethylated (−Me) or *Sss*I-methylated (+Me) 5S-pmbs0. Nucleosomes (0.9 pmol) were incubated with: 0 (lanes 1 and 8), 0.375 (lanes 2 and 9), 0.75 (lanes 3 and 10), 1.5 (lanes 4 and 11), 3.0 (lanes 5 and 12), 6.0 (lanes 6 and 13) and 12 (lanes 7 and 14) pmol of MeCP2. (C) Same as in (B), but using nucleosomes reconstituted onto 5S-pmbs23. Samples were incubated with 0 (lanes 1 and 7), 0.75 (lanes 2 and 8), 1.5 (lanes 3 and 9), 3.0 (lanes 4 and 10), 6.0 (lanes 5 and 11) and 12 (lanes 6 and 12) pmol of MeCP2. (D) Gel shift assay of nucleosomes (0.9 pmol) reconstituted onto methylated 5S-pmbs −3, −10, −23 and −37 (see Fig. S1D). Samples were incubated with: 0 (lanes 1, 5, 9 and 13), 0.75 (lanes 2, 6, 10 and 14), 1.5 (lanes 3, 7, 11 and 15) and 3.0 (lanes 4, 8, 12 and 16) pmol of MeCP2. *C1* and *C2*: MeCP2–DNA complexes; *D*: free DNA; *N*: nucleosome. The C2 complexes appear only upon saturation of the linker preferential binding site at very high ratios of MeCP2:nucleosome with likely additional binding to less preferred sites within the nucleosome region.

Fig. 3B and C). In order to assess if, like histone H1, MeCP2 has a preference for the site where the linker DNA enters and exits the nucleosome, a preferential methylation binding site (pmbs) was moved to different positions along the linker DNA (Fig. 1B and Fig. 3A). Fig. 3D shows that the proximal site (pmbs37) (six nucleotides away from the octamer) is clearly a preferred binding site, similar to what is observed for linker histones in the chromatosome [24,25]. Like linker histones, MeCP2 exhibits a preferential binding for cruciform DNA structures that mimic the organization of DNA at the entry and exit sites of the nucleosome in the chromatosome structure [26].

To ensure that the binding affinity of MeCP2 for the different pmbs observed in Fig. 3D was not affected by the differences in the sequence of the artificially created pmbs sites shown in Fig. 1B, a control experiment was performed. The higher affinity exhibited by pmbs37 is unlikely due to this, as it has the longest nucleotide span between the CpG and the adjacent $[A/T]_{\geqslant 4}$ [14]. Nevertheless, we repeated this experiment using DNA constructs in which all the other pmbs have an identical sequence to that of the pmbs37 (see Fig. S2A–C). The results are shown in Fig. S2C and clearly show that the difference in MeCP2 binding affinity to nucleosomes depends on the position of the pmbs rather than its particular sequence.

3.2. Binding of MeCP2 to chromatin does not depend on the presence of the N-terminal tail of H3 and it is not affected by the presence of histone H1 or core histone acetylation

A recent study has provided evidence that in the chromatosome-like structure resulting from the interaction of the nucleosome core particle with MeCP2, MeCP2 is in close proximity to histone H3. It was suggested that a potential interaction between these two proteins may involve the N-terminal tail of histone H3 [12]. The results of Fig. 4A show that the binding of MeCP2 to the nucleosome is slightly favoured by the lack of the N-terminal tail of histone H3 (compare shifts observed in lanes 3 and 4 with those of lanes 7 and 8). This indicates that while MeCP2 may interact in a region of the linker DNA that partially overlaps with that occupied by the N-terminal tail of H3 in the chromatosome, the presence or absence of the H3 N-terminal tail is not a major determinant of MeCP2 binding. This result is not surprising as the binding of linker histones to nucleosomes does not depend upon the amino termini of core histones [27]. It has been shown that the N-terminal tail of histone H3 and linker histones have structural redundancy in determining chromatin conformational stability. It is possible that MeCP2 exhibits a similar redundancy.

Similarly, acetylation of core histones does not appear to strongly influence linker histone binding to the nucleosome [28]. Hence, it would not be expected to have any major effect

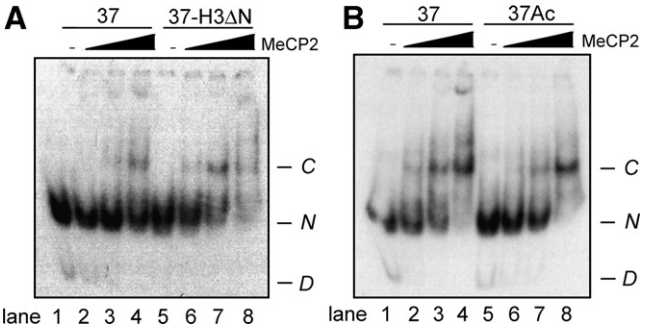

Fig. 4. Neither removal of the H3 N-terminal tail nor histone acetylation affects the binding of MeCP2 to the nucleosome. (A and B) Gel shifts of nucleosomes reconstituted onto methylated 5S-pmbs37 (A) using histone octamers with trypsinized H3 (37-H3ΔN) [see Fig. S1B, lane T] or (B) using acetylated histone octamers (37Ac) (see Fig. S1B and C, lanes N and T). Nucleosomes (2 pmol in A and 1.3 pmol in B) were incubated with 0 (lanes 1 and 5), 0.75 (lanes 2 and 6), 1.5 (lanes 3 and 7) or 3.0 (lanes 4 and 8) pmol MeCP2. *C, D, N*: same as in Fig. 3.

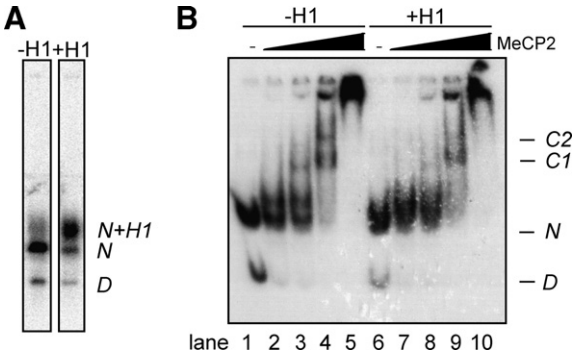

Fig. 5. MeCP2 binds to nucleosomes independently of the presence of linker histones. (A) Agarose gel of nucleosomes reconstituted onto methylated 5S-pmbs37 DNA without (−H1) or with (+H1) histone H1. Agarose gels were used to monitor the binding of H1 to nucleosomes as a clear shift (*N + H1*) can only be observed in this type of gels but not in native-PAGE [29,37]. (B) The appropriate *N + H1* complexes such as that shown in (A) were then used in further MeCP2 gel shift using native-PAGE. Nucleosomes lacking (−H1) or containing (+H1) histone H1 (see also Fig. S3) upon addition of: 0 (lanes 1 and 6), 0.75 (lanes 2 and 7), 1.5 (lanes 3 and 8), 3.0 (lanes 4 and 9) and 6.0 (lanes 5 and 10) pmol of MeCP2. *C, D* and *N* are as in previous figures.

on MeCP2 binding to the nucleosome in Fig. 4B. Indeed, no major differences were observed between MeCP2 binding to nucleosomes reconstituted with either non-acetylated or acetylated core histone octamers (Fig. 4B).

Finally, our *in vitro* studies show that MeCP2 can bind to the nucleosome in the presence of histone H1. To perform this, nucleosomes were first titrated (results not shown) with histone H1 using agarose gel electrophoresis [29] to determine the amount of histone H1 required to shift the nucleosome to a nucleosome complex consisting of approximately 1 molecule of H1 per nucleosome (Fig. 5A, lane +H1, *N + H1*). Beyond this point, a complex with two molecules of H1 per nucleosome and higher aggregates were observed (results not shown) [29]. Under these chosen experimental conditions, H1 asymmetrically protects the DNA and overlaps with the binding site of MeCP2 in the 5S-pmbs37 {Fig. S3 (see also [18])}. The MeCP2 gel shifts using the N + H1 complexes are shown in Fig. 5B and show an almost indistinguishable pattern. Due to the large excess of non-radioactively labeled competitor DNA used in the nucleosome reconstitution experiments (see supplemental experimental procedures), it is almost impossible to perform histone H1/MeCP2 competition experiments that

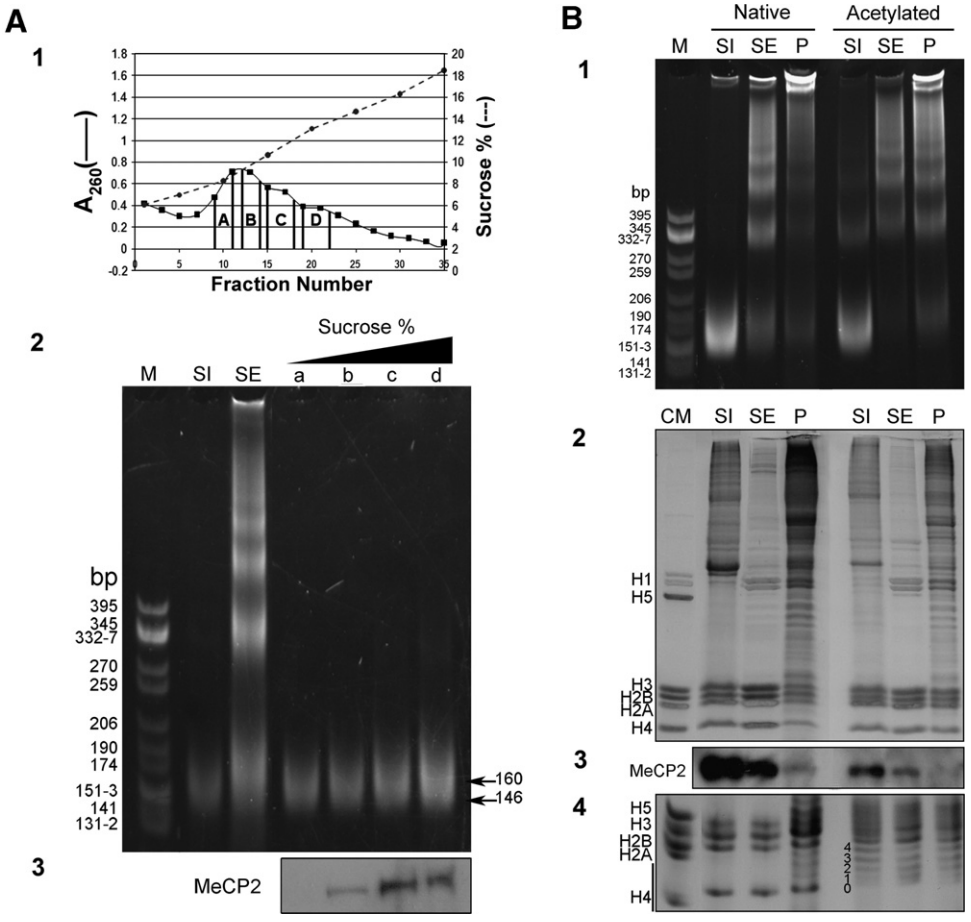

Fig. 6. MeCP2 distribution in HeLa S3 cell chromatin. (A) (1) Sucrose gradient fractionation of the nucleosome SI fraction obtained from HeLa cells. The absorbance at 260 nm (A_{260}) and the sucrose concentration of collected fractions are shown. (2) DNA composition of SI nucleosomes of the (a–d) shown in (1) Starting DNA from SI and SE samples are shown in comparison. The two arrowheads point to the range (146–160 bp) of the DNA of the SI nucleosomes. (3) Western blot analysis of the MeCP2 present in the sucrose gradient fractions. (B) (1) Native-PAGE of the DNA composition of the SI, SE and P fractions generated by micrococcal nuclease digestion of native and butyrate-treated HeLa S3 nuclei. M: pBR322-*Cfo*I-digested marker. (2) SDS–PAGE protein analysis of fractions SI, SE and P. (3) Western blot analysis of the gel shown in (2) using an α-MeCP2 antibody. (4) AU–PAGE of SI, SE and P fractions. The vertical line on the left of B4 indicates the range of H4 isoforms with 0–4 denoting the acetylated forms of H4.

allow us to monitor the displacement of one protein by the other. Nevertheless, the shifted band in lane 9 of Fig. 5 and the strong similarity of the MeCP2 shifts both in the absence and in the presence of histone H1 suggest that histone H1 does not prevent MeCP2 from binding. An N + H1 band is not observed in lane 6 (Fig. 5B) due to the resolving limitations of native-PAGE. Otherwise, this complex is observed using agarose gel electrophoresis (Fig. 5A).

3.3. MeCP2 binds to the nucleosome proximal region of the linker in vivo and is found predominantly associated with histone H1-depleted nucleosomes in HeLa S3 cells

The relevance of the results described in the preceding sections ultimately depends on whether they are a true reflection of what occurs *in vivo*. To this end, we used HeLa cells and a simple micrococcal nuclease digestion that yields a histone H1-depleted soluble fraction (SI) consisting of a mixture of 146–160 bp nucleosomes and an H1-containing fraction (SE) consisting of >168 bp nucleosomes and nucleosome oligomers (Fig. 6A and B). Most of the MeCP2 unexpectedly eluted with the SI fraction, and the result was very similar when the cells were grown in the presence of the histone deacetylase inhibitor sodium butyrate (Fig. 6B2–B3).

This suggests, in agreement with the *in vitro* data, that core histone acetylation does not influence the way MeCP2 binds to the nucleosome. Furthermore, sucrose gradient fractionation of the SI nucleosomes (Fig. 6A1) showed that MeCP2 binding to nucleosomes was enhanced when DNA lengths were within a 10 bp range above 146 bp (Fig. 6A) in striking similarity to what was observed *in vitro* in Fig. 3C.

The SI and SE fractions represent the less tightly packed euchromatin and facultative heterochromatin. Under the digestion conditions used, these fractions comprised: SI (11%), SE (26%), P (63%) in the untreated HeLa S3 cells and SI (15%), SE (13%), P (72%) in the butyrate-treated fraction. The lower amount of MeCP2 present in the butyrate-treated HeLa cells chromatin is also intriguing, but agrees well with the selective loss of DNA methylation that has been observed upon treatment of different cells with histone deacetylase inhibitors [30,31]. Preliminary data from our lab using HpaII and MspI digestions of nucleosomal DNA from the SI fraction indicates a 6.7% decrease in the extent of methylation in the fraction obtained from sodium butyrate treated cells compared to untreated cells (see Fig. S4 and the supplementary experimental procedures). Work is currently in progress in our lab to analyze these observations further.

3.4. Conclusions

The results presented here collectively show that MeCP2 binds to the linker DNA very close to the DNA entry and exit sites of the nucleosome, both *in vitro* and *in vivo*. In contrast to linker histones [32], MeCP2 needs the DNA to be methylated. *In vivo*, the MeCP2-containing HeLa cell nucleosomes are readily digested, suggesting that most of these nucleosomes occur outside of the tightly folded constitutive heterochromatin [33]. Intuitively, such CpG pmbs in the proximity of a nuclease-accessible nucleosome are more likely to occur in CpG rich regions in facultative heterochromatin where MeCP2 (unlike linker histones) could provide a distinctive binding site for heterochromatin protein 1 (HP1) [34] or other chromatin transacting factors. The *in vivo* observations described in this paper are in excellent agreement with the recently published large-scale mapping of neuronal MeCP2 binding sites showing that 63% of MeCP2 bound promoters are in actively expressed genes [35].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.03.005.

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